EXOGENOUSLY APPLIED PROLINE MITIGATES ADVERSE EFFECTS OF SALT STRESS IN WHEAT (*TRITICUM AESTIVUM*) THROUGH DIFFERENTIAL MODULATION OF ANTI-OXIDATIVE DEFENCE SYSTEM AND OSMOLYTES ACCUMULATION

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(Received 14th May 2024; accepted 23rd Sep 2024)

Abstract. Soil salinity is a major environmental problem all over the globe. This issue is of great concern and needs special attention as it reduces fertility of the agricultural land and retards seedlings development and growth. Recently various techniques are under consideration to reclaim salt affected soils. Imparting tolerance against salt stress by employing organic supplements is one of the useful methods. In this study, effects of exogenously applied proline (50 mM and 100 mM) on the germination, growth and biochemical attributes of two cultivars of *Triticum aestivum* L., namely Aanj 2017 and Faisalabad 2008 at the different levels of salt stress (50 mM and 100 mM) were examined. Plants were randomly arranged in control groups (no salt and proline treatment), different levels of salinity and proline, and their combined application. The salt stress (NaCl) suppressed the parameters related to germination, growth and biochemical compositions in both genotypes especially in Faisalabad 2008 was badly stunted. Exogenously applied proline has tremendously counteracted the adverse effects of salinity in both genotypes by upregulation of antioxidant defense system, promoting the efficiency of photosynthetic pigments and flavonoids, improvements in uptake of mineral ions and water, however, the performances of Aanj 2017 surpassed in the presence of proline.
Keywords: *salinity, wheat, foliar spray, proline, organic osmolytes, flavonoids, antioxidants, yield*

Introduction

Several environmental factors affect crop production. Among these stresses, soil salinity severely affects physical and chemical properties of soil and causes substantial yield losses to plant cultivars. It is estimated that 1/3 of the land throughout the world is degraded by soil salinity (Ramadoss et al., 2013). This

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2206_59295947

accounts for about 800 million ha of the land all over the world where high levels of salts in soil badly affect mineral profile and microbial activity as well as water-relations in plants According to expert agriculturists and soil engineers, about 50% of cultivable land would become unfertile (for the most parts in the developing countries) if this problem is not combated in time (Moukhtari et al., 2020). Recent advances in technology for soil manipulation and excessive use of chemical fertilizers along with bad quality irrigation water for greater crop production had intensified the process of salinity (Kalhoro et al., 2016). If the rate of soil degradation due to salinity soil salinity continues at this pace, it would impose a serious threat to food security in future.

Salinity disturbs the metabolism of plants by interfering with the physiological processes in two ways. Firstly, it may imbalance the osmotic regulations as the salt concentration in soil is far beyond the tolerance level. Secondly, it may mediate the accumulation of heavy metals or cytotoxic chemicals to a drastic level which ultimately results in reduction of growth and premature cell death (Abbas et al., 2013). Salinity induces severe disorders in plants including reduction in stomatal conductance, decrease in photosynthetic efficiency, and imbalance in ions and nutrient uptake. Finally, fluctuations in morphological patterns such as stunted growth of roots and shoots, less leaves and fruit production, and disorganization in metabolic processes and signal transduction are all associated with the exaggerated saline conditions (Munns et al., 2006).

To mitigate the toxic effects of salinity, plants accumulate excessive amounts of proline. It is also applied to the plants endogenously and exogenously (Heuer, 2010). Proline is the common osmolyte that provides resistance against the effects of salinity by adjusting the osmotic potential of cells and regulating membrane and enzymatic activities. It also works as nitrogen storage compound and signal transducer, and shields the cells from the oxidative stress of reactive oxygen species. Among these, foliar spray of proline has been shown to develop tolerance to salinity stress in plants. It triggers various biochemical and physiological processes like enhancement of water and mineral absorption and acceleration of gaseous exchange resulting in faster germination rate, better morphological attributes, and greater biomass production. Proline optimizes the chemical activities of antioxidants and suppresses the uptake of Na^+ and Cl^- ions so that further accumulation of salt could be culminated (Moukhtari et al., 2020). Under osmotic stress, foliar application of proline affects the biochemical and physiological traits and upsurges the rate of the seedling's growth in saline conditions (Mahboob et al., 2016).

Wheat (*Triticum aestivum* L.) is one of the most important salt tolerant crops, however, several genotypes are susceptible to the salt stress. Delay in seedling emergence and inhibition in germination are the preliminary disorders associated with salinity. These negative effects are described in terms of biological disarrays such as deleterious effects on the germination phase and reduction in mineral uptake (Ashraf et al., 2018). Reduction in water-uptake is regarded as the principle limiting factor observed under salt stress which ultimately prevents normal growth and development of seedlings (Arslan and Ashraf, 2012). Salinity imposes several negative impacts on the metabolic machinery such as reduction in concentration of chlorophyll, greater reactivate oxygen species (ROS) content and ion toxicity (Saddiq et al., 2019). Eventually, the photosynthesis rate of the plants decreases and premature leaf senescence occurs (Hussain et al., 2018).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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In view of the useful effects of proline like mitigating salt stress and growth promotion, it was hypothesized that exogenous application of proline could be strengthening the defense system by up-regulating the anti-oxidative activities and accumulation of the osmolytes in plants. The purpose of the current research experiment was to evaluate the possible mechanisms involved in mitigating salt stress by applying proline exogenously on two wheat cultivars.

Materials and methods

Experimental layout and treatment levels

Seeds of two wheat cultivars, Faisalabad 2008 and Aanj 2017, were obtained from the Ayyub Agricultural Research Institute in Faisalabad, Pakistan. The seeds were sterilized and sown in plastic pots filled with fertile, silt-loam soil. A total of 54 pots were arranged in a completely randomized design (CRD) to assess the impact of different treatments, including salinity, proline, and combined salinity and proline applications, as well as a control group. The experimental design comprised a control group (T0) without salt stress or proline application, and eight treatment groups with varying levels of NaCl and proline. The treatment groups were:

T1: 50 mM Proline application
T2: 100 mM Proline application
T3: 50 mM NaCl application (salt stress)
T4: Combined application of 50 mM NaCl and 50 mM Proline
T5: Combined application of 50 mM NaCl and 100 mM Proline
T6: 100 mM NaCl application (salt stress)
T7: Combined application of 100 mM NaCl and 50 mM Proline
T8: Combined application of 100 mM NaCl and 100 mM Proline

The NaCl solution was applied to the soil before seed sowing. The required levels of proline were prepared in water and tween-20 was added as surfactant. The solution was sprayed to 21-days old plants in the early morning when the dew evaporated but sun was still low. A 10 ml solution was sprayed to completely wet all leaves. Each treatment was given in triplicate.

Collection of data

Samples were taken from the 75 days older plants and various morphological, physiological and biochemical parameters were investigated.

Morphological parameters

The plants were uprooted carefully without damaging the roots and shoot systems. After removing soil from the samples, different morphological parameters including number of leaves, leaf area, plant height, fresh weight of shoot and root, and length of root and shoot, were measured. After measurement of the fresh samples, they were transferred to oven at 62°C for 7 days to measure the dry biomass. Samples for the determination of physiological and biochemical parameters were preserved in refrigerator.

Leaf area was calculated using following formula:

$$\text{Area of leaf} = \text{Length of leaf} \times \text{Width of leaf} \times \text{C.F.}\,(\text{correction factor} = 0.68)$$

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Photosynthetic pigments

Arnon's (1949) protocol was followed to measure chlorophyll while that of Davis (1965) for carotenoid pigments. A 0.1 g of fresh leaf sample was kept overnight in 80% acetone. The extract was filtered using filter paper and centrifuged at 10,000 rpm for 5 min. The absorbance of samples for chl. *a*, *b*, and carotenoids was measured through UV/Visible Spectrophotometer at 663 nm, 645 nm, and 480 nm, respectively. Concentration of these pigments was measured by adopting standard formulae.

Antioxidant enzyme extraction

The activities of antioxidant enzymes, including catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD), were assayed by extracting fresh leaf samples in 50 mM chilled phosphate buffer (pH 7.8). The extract was then centrifuged at 15,000 rpm for 15 min, and the resulting supernatant was used to prepare reaction mixtures for the measurement of various enzymatic activities.

Peroxidase and catalase (POD and CAT)

Catalases and peroxidases were measured by referring to the Maehly and Chance's protocol (1955). Reaction mixture of peroxidase contained 50 µL enzyme extract, 750 µl phosphate buffer (pH7.8), 100 µL guaiacol and 100 µL H_2O_2. The change in optical density was recorded at 470 nm at the interval of 20 s. The POD activity was quantified as an absorbance change of 0.01 units/min. The catalase assay mixture comprised 100 µL enzyme extract, 1.9 mL phosphate buffer (pH 7.8), 100 µL guaiacol, and 1 mL H_2O_2. Optical density was measured at 240 nm at 20-s intervals. One unit of CAT activity was equivalent to an absorbance change of 0.01 units/min.

Superoxide dismutase (SOD)

SOD activity was quantified using the Giannopolitis and Ries (1977) method, with NBT as the substrate. The reaction mixture contained potassium phosphate buffer, enzyme extract, NBT, riboflavin, methionine, and H_2O_2. After 15 min of illumination (at 350 nm), SOD concentration was measured spectrophotometrically at 560 nm. One unit of SOD activity was equivalent to 50% NBT photoreduction.

Ascorbic acid peroxidase (APX)

The Rao et al. (1996) protocol was followed to measure the activity level of APX. The reaction mixture for this analysis contained the enzyme extract equivalent to 150 µg, 100 mM potassium phosphate buffer (pH 7.8), 0.5 mM ASC, and 0.2 mM H_2O_2. The levels of APX were recorded at 290 nm for 1 min of activity.

Estimation of total soluble proteins (TSP)

Total soluble proteins were estimated by Bradford method (1976). A sample of 0.5 g from fresh leaf tissue was ground in 5 mL of 50 mM chilled phosphate buffer (pH 7.8). The extract centrifuged at 1,5000 rpm for 15 min. A 0.1 µL of the supernatant was mixed with 5 mL of Bradford reagent and incubated at 37°C along with the blank for 10-15 min. Absorbance of the total soluble proteins was taken at 595 nm with the help of spectrophotometer (IRMECO U2020).

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Proline determination

Proline content was determined using the method described by Bates et al. (1973). A 0.5 mL fresh leaf sample was homogenized in 10 mL of 30% sulfosalicylic acid and filtered. The filtrate was then mixed with 2 mL of ninhydrin and 2 mL of glacial acetic acid. The mixture was heated in a water bath for 60 min, allowed to cool, and then extracted with 4 mL of toluene. The absorbance of the chromophoric aqueous phase was measured at 520 nm. The proline content was quantified by referencing a standard curve generated from known proline concentrations.

Total free amino acids (TFA) estimation

The reaction mixture for the quantification of total free amino acids was prepared by method of Vartainan et al. (1992). After extraction, the concentration of the free amino acids was measured by following Yemm and Cocking (1955) protocol. A leaf sample of 500 µL were inoculated with the 500 µL solution of ninhydrin (2%) and 500 µL solution of pyridine (10%) in test tube. Incubate in water bath (95°) for 30 min. Add 10 mL distilled water in the test tube. Take readings at 570 nm when cool. Phosphate buffer used as a blank. The final quantity of P was measured by comparing it with known standards.

Determination of flavonoids

The plant sample (0.1 g) was ground in 80% acetone solution and 4 mL of distilled water for the extraction of flavonoids (Zhishen et al., 1999). To prepare the reaction mixture, 1 ml of extracted solution added in the test tube with 0.6 mL of 5% $NaNO_2$ and 2 mL of 10% $AlCl_3$. After 5 min interval, 2 mL sodium hydroxide solution (1 M) was added into the test tube. To make the sample dilute, 2.4 mL distilled water was poured into the reaction mixture. The concentration of flavonoids was calculated by directing a wavelength of 510 nm with the help of spectrophotometer.

Determination of mineral nutrients

The concentration of mineral ions sodium (Na^+), potassium (K^+), and calcium (Ca^{2+}) were measured by following the protocol of Cresser and Parsons (1979). The samples were prepared by the help of acid digestion and readings were recorded in comparison to the blank (H_2O_2) with the help of flame photometer.

Statistical analysis

Analysis of variance (ANOVA) was applied on obtained data by using Co-Stat computer programs. The values were compared using least significant differences (LSD) and the significant difference was expressed as different alphabets on bar graphs. R Studios was used for multivariate analysis (R Core Team, 2021). The correlograms, heatmaps and principal component analysis were performed in R (R i386 4.0.5).

Results

Salinity imposed negative effects on the germination and growth of wheat seedlings as noticed by the significant reduction in morphological parameters like lower fresh and dry weights in shoot and root and shorter seedling heights. Salt stress proved detrimental at both low (50 mM) and high (100 mM) levels. However, plants grown

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under exogenously applied proline showed better development and sprouted vigorously in saline conditions. Application of proline resulted in pronounced outcomes as illustrated by the better growth patterns i.e., greater biomass production and taller shoot and root lengths (*Fig. 1*). Extremely low performance in view of the overall growth efficiencies were observed in the plants under salt stress of 100 mM level, in contrast, the maximum growth dynamics were obtained in the genotype Aanj 2017 at proline treatment level of 100 mM even under the high salt level. As it was directly revealed from various morphological parameters, the salinity as for the treatment level of 100 mM has severely reduced the plant height, number of leaves, and leaf zone. Though both wheat cultivars i.e., Aanj 2017 and Faisalabad 2008 showed significant improvement in the salt stress by the foliar application of proline, the former cultivar exhibited prominent performance (*Fig. 2*).

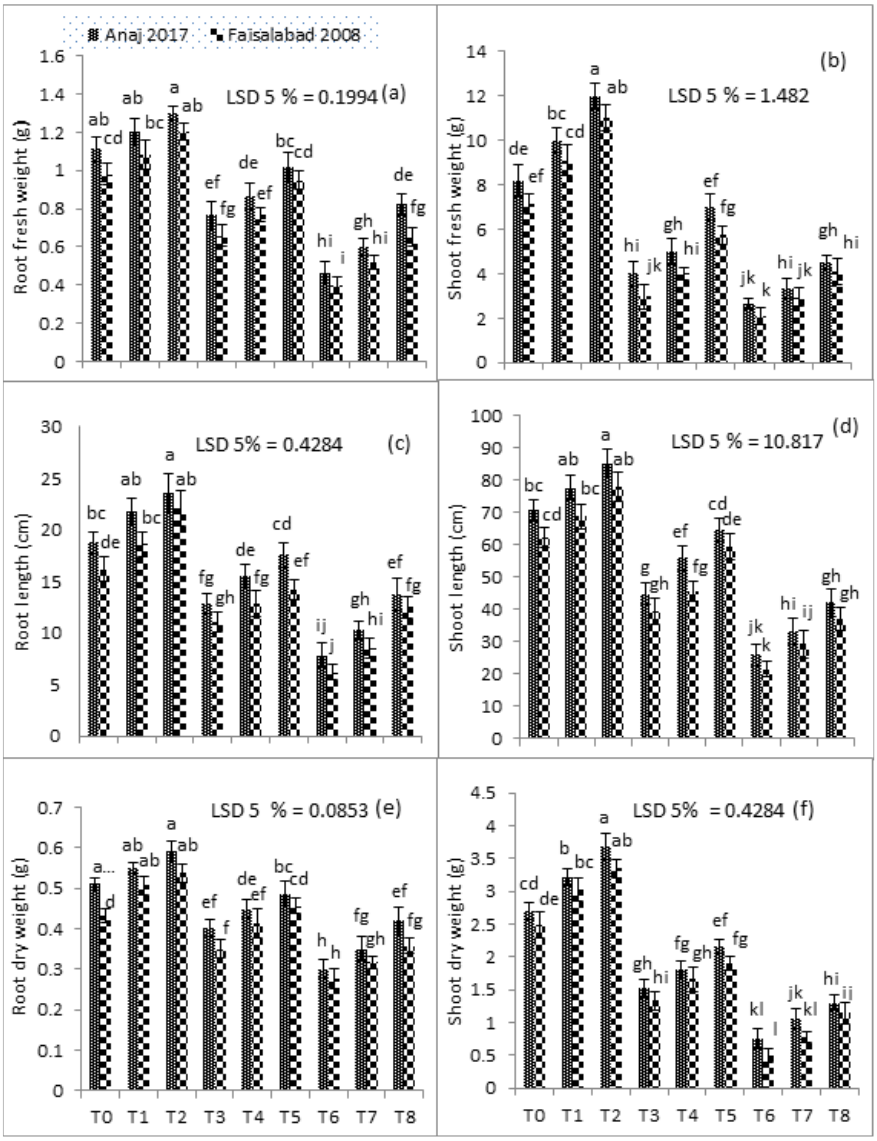

Figure 1. *Effect of exogenously applied proline on (a) root fresh weight, (b) shoot fresh weight, (c) root length, (d) shoot length, (e) root dry weight and (f) shoot dry weight of two wheat (Triticum aestivum L.) varieties under salt stress. T_0 = Control, T_1 = 50 mM Proline, T_2 = 100 mM Proline, T_3 = 50 mM NaCl, T_4 = 50 mM NaCl + 50 mM Proline, T_5 = 50 mM NaCl + 100 mM Proline, T_6 = 100 mM NaCl, T_7 = 100 mM NaCl + 50 mM Proline, T_8 = 100 mM NaCl + 1 00 mM Proline*

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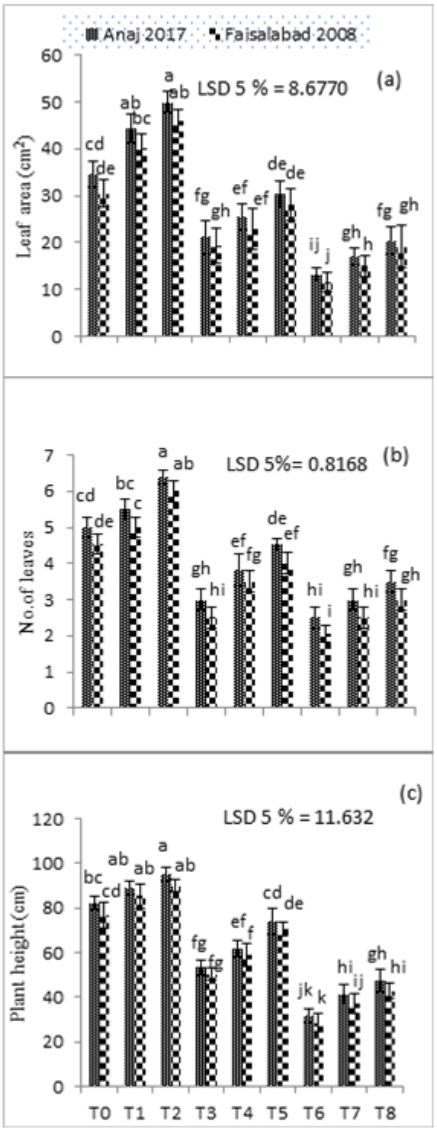

Figure 2. *Effect of exogenously applied proline on (a) leaf area, (b) number of leaves and (c) plant height of two wheat (Triticum aestivum L.) varieties under salt stress. T_0 = Control, T_1 = 50 mM Proline, T_2 = 100 mM Proline, T_3 = 50 mM NaCl, T_4 = 50 mM NaCl + 50 mM Proline, T_5 = 50 mM NaCl + 100 mM Proline, T_6 = 100 mM NaCl, T_7 = 100 mM NaCl + 50 mM Proline, T_8 = 100 mM NaCl + 100 mM Proline*

Analysis of the photosynthetic pigments like concentration of chlorophyll a and b, total chlorophyll and carotenoids contents, and, flavonoids confirmed a severe inhibitory effect of salinity that were significantly alleviated by of the application of proline in both genotypes of wheat. Higher chlorophyll and flavonoid contents were observed in the 100 mM proline treatment under control as well as in salt stress. Faisalabad 2008 produced lower amounts of photosynthetic biomolecules in response to the salinity while Aanj 2017 conceded greatest contributions in regard to the photosystems under the influence of externally provided proline (*Fig. 3*).

The externally applied proline brought up significant enhancement in the internal proline and free amino acid levels. Statistical analysis presented the significant difference between the data obtained from the internal proline and free amino acids contents under

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salt stress and the proline treatment. Plants produced lower amounts of these compounds in the presence of excessive salt concentrations while higher amounts were observed in the samples obtained from the plants under exogenously applied proline. Genotype Aanj 2017 was somewhat better than Faisalabad 2008 with respect to these metabolites in all the treatment levels. Here, significant differences were only proven for total free amino acids in T2 and T3 treatment (*Fig. 4*). As illustrated in *Figure 5*, the biochemical contents like TSP, TSS and anthocyanin were also reduced under saline conditions but proline application elevated their concentrations in even under salt treated plants. However, based on *Figure 5*, it was observed there was no significant difference between the genotypes at T2 treatment. On a similar pattern, higher salt levels and proline application significantly influenced the activities of antioxidant compounds.

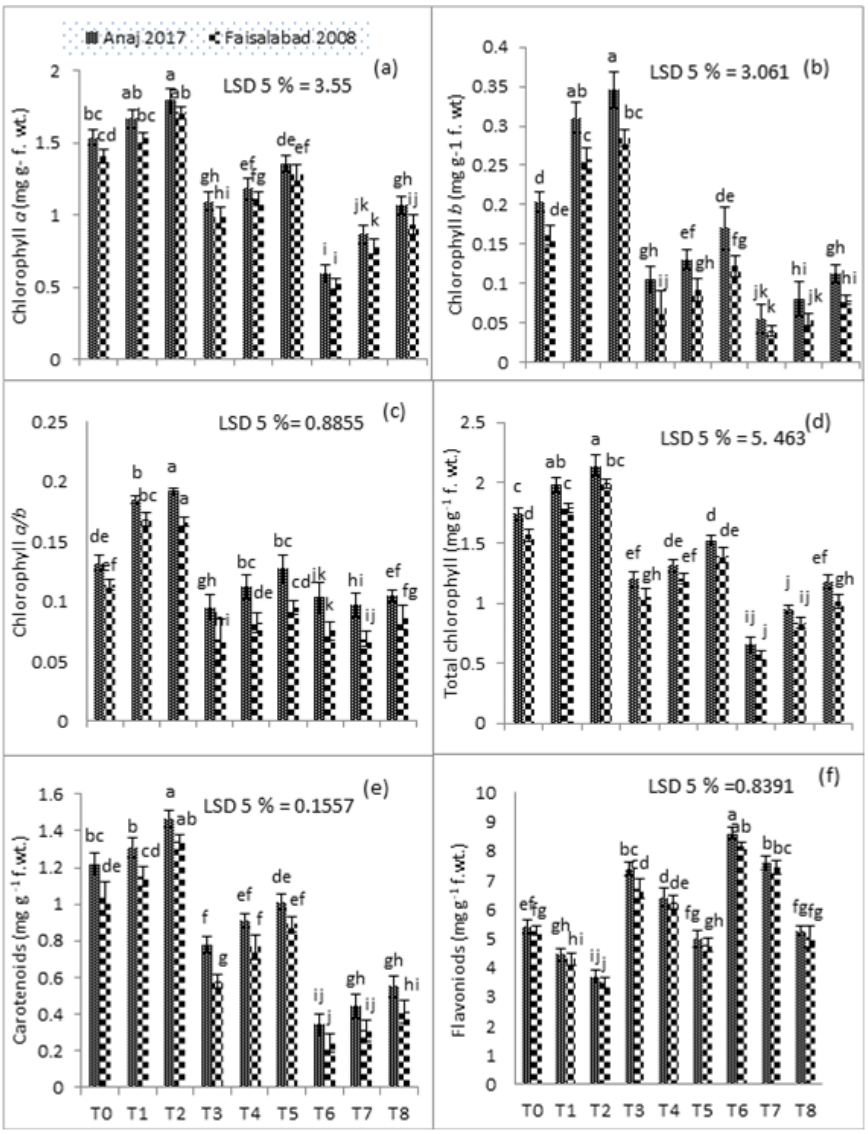

Figure 3. *Effect of exogenously applied proline on (a) chlorophyll a, (b) chlorophyll b, (c) chlorophyll a/b, (d) total chlorophyll, (e) carotenoids and (f) flavonoids of two wheat (Triticum aestivum L.) varieties under salt stress. T_0 = Control, T_1 = 50 mM Proline, T_2 = 100 mM Proline, T_3 = 50 mM NaCl, T_4 = 50 mM NaCl + 50 mM Proline, T_5 = 50 mM NaCl + 100 mM Proline, T_6 = 100 mM NaCl, T_7 = 100 mM NaCl + 50 mM Proline, T_8 = 100 mM NaCl + 100 mM Proline*

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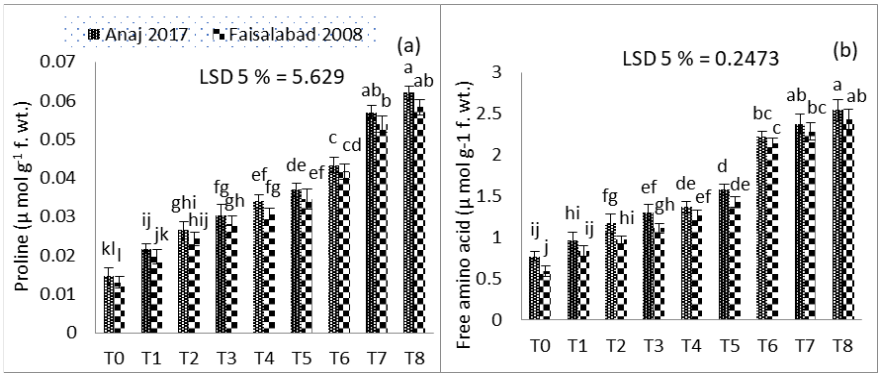

Figure 4. *Effect of exogenously applied proline on (a) proline and (b) total free amino acids (TFFA) of two wheat (Triticum aestivum L.) varieties under salt stress. T_0 = Control, T_1 = 50 mM Proline, T_2 = 100 mM Proline, T_3 = 50 mM NaCl, T_4 = 50 mM NaCl + 50 mM Proline, T_5 = 50 mM NaCl + 100 mM Proline, T_6 = 100 mM NaCl, T_7 = 100 mM NaCl + 50 mM Proline, T_8 = 100 mM NaCl + 100 mM Proline*

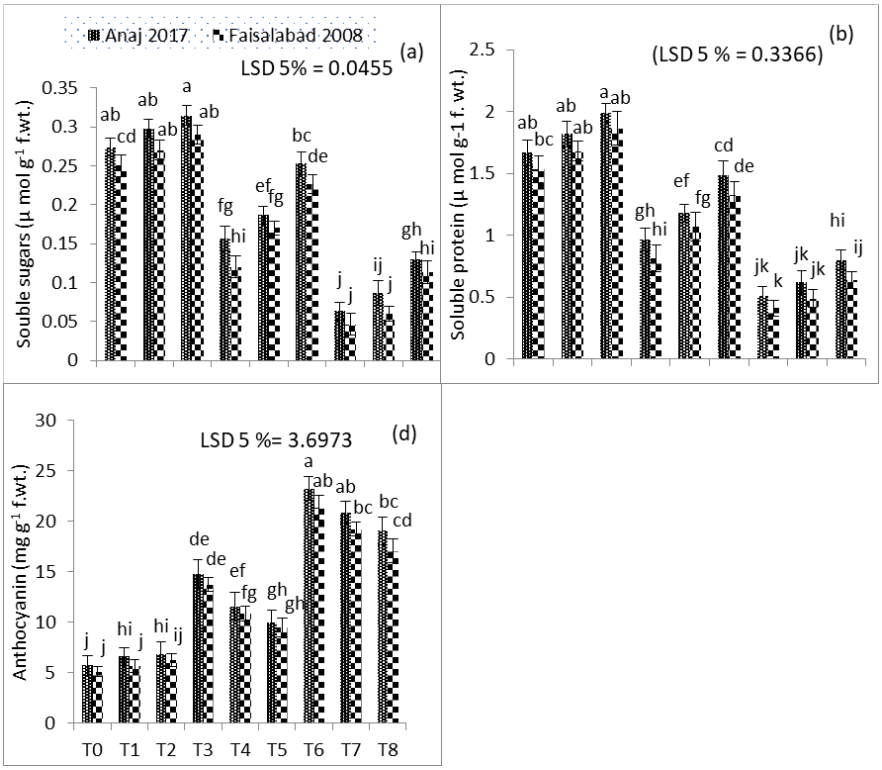

Figure 5. *Effect of exogenously applied proline on (a) total soluble sugars (TSS), (b) total soluble protein (TSP) and anthocyanin of two wheat (Triticum aestivum L.) varieties under salt stress. T_0 = Control, T_1 = 50 mM Proline, T_2 = 100 mM Proline, T_3 = 50 mM NaCl, T_4 = 50 mM NaCl + 50 mM Proline, T_5 = 50 mM NaCl + 100 mM Proline, T_6 = 100 mM NaCl, T_7 = 100 mM NaCl + 50 mM Proline, T_8 = 100 mM NaCl + 100 mM Proline*

Enzymatic activities of SOD, POD, CAT and APX were the higher in 100 mM salt treatment in Aanj 2017 without proline application. Under the exogenous application of proline, the concentration of antioxidant like total phenolic content increased when plants

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were exposed to 100 mM NaCl levels. Higher total phenolic compounds were observed in the genotype Faisalabad 2008 in the absence of salinity (*Fig. 6*).

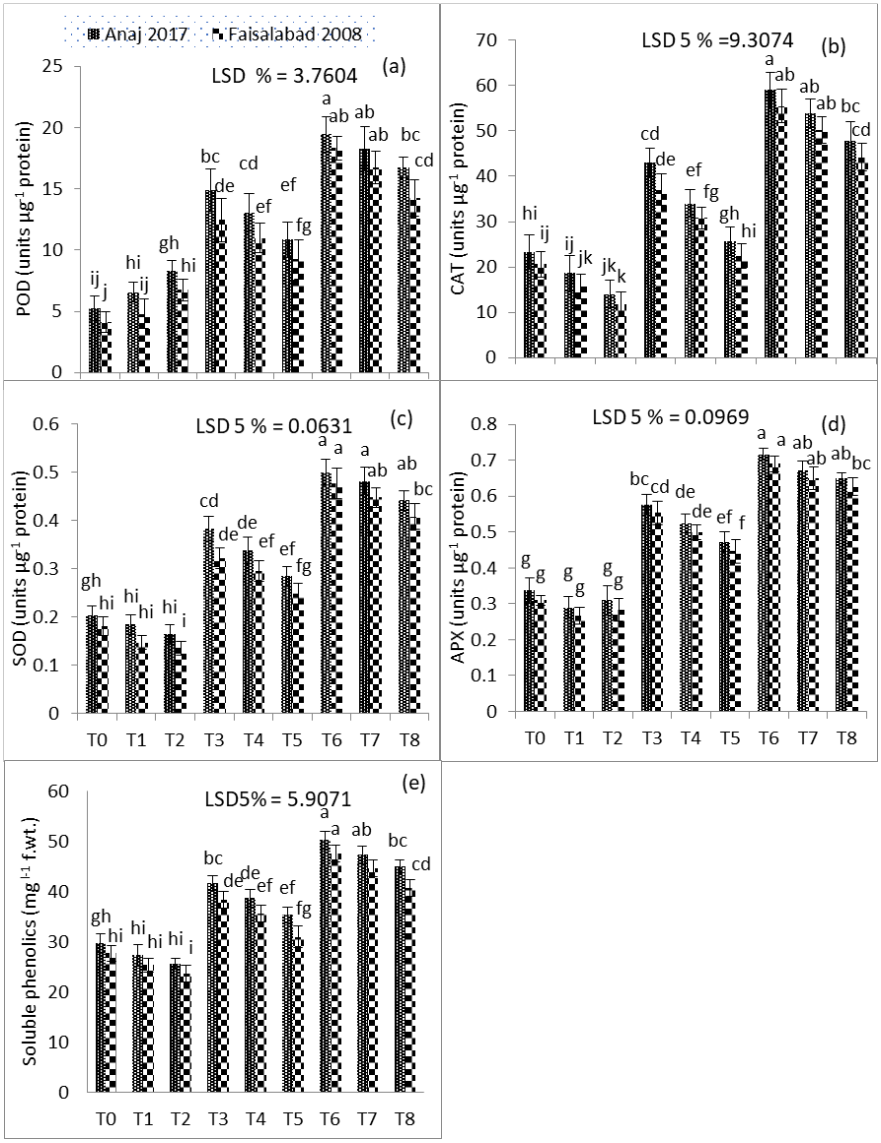

Figure 6. *Effect of exogenously applied proline on (a) peroxidase (POD), (b) catalase (CAT), (c) superoxide dismutase (SOD), (d) ascorbate peroxidase (APX) and (e) total soluble phenolics (TPh) of two wheat (Triticum aestivum L.) varieties under salt stress. T_0 = Control, T_1 = 50 mM Proline, T_2 = 100 mM Proline, T_3 = 50 mM NaCl, T_4 = 50 mM NaCl + 50 mM Proline, T_5 = 50 mM NaCl + 100 mM Proline, T_6 = 100 mM NaCl, T_7 = 100 mM NaCl + 50 mM Proline, T_8 = 100 mM NaCl + 100 mM Proline*

Salinity adversely affected the growth attributes by increasing the accumulation of sodium ions but decreasing other ions (*Fig. 7*). These important mineral ions like calcium and potassium ions decreased in 100 mM treated plants of the both the genotypes. However, proline treated plants were observed with an uprise in these essential mineral ions accumulation in both salt-stressed and non-stressed groups with a concurrent decrease in internal sodium concentration.

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Correlogram

The correlogram were constructed for Pearson's correlation coefficient (r) showing relationship between morpho-physiological attributes of wheat varieties Aanj 2017 (a) and Faisalabad 2008 (b) in response to salt stress under exogenously applied proline (*Fig. 8*). In both cultivars, most of the physiological and biochemical parameters were positively correlated with growth. However, antioxidants (CAT, POD, SOD, APX), flavonoids, anthocyanins, phenolics, and root Na were negatively correlated. The total free amino acids was non-significantly correlated (*Fig. 8a, b*).

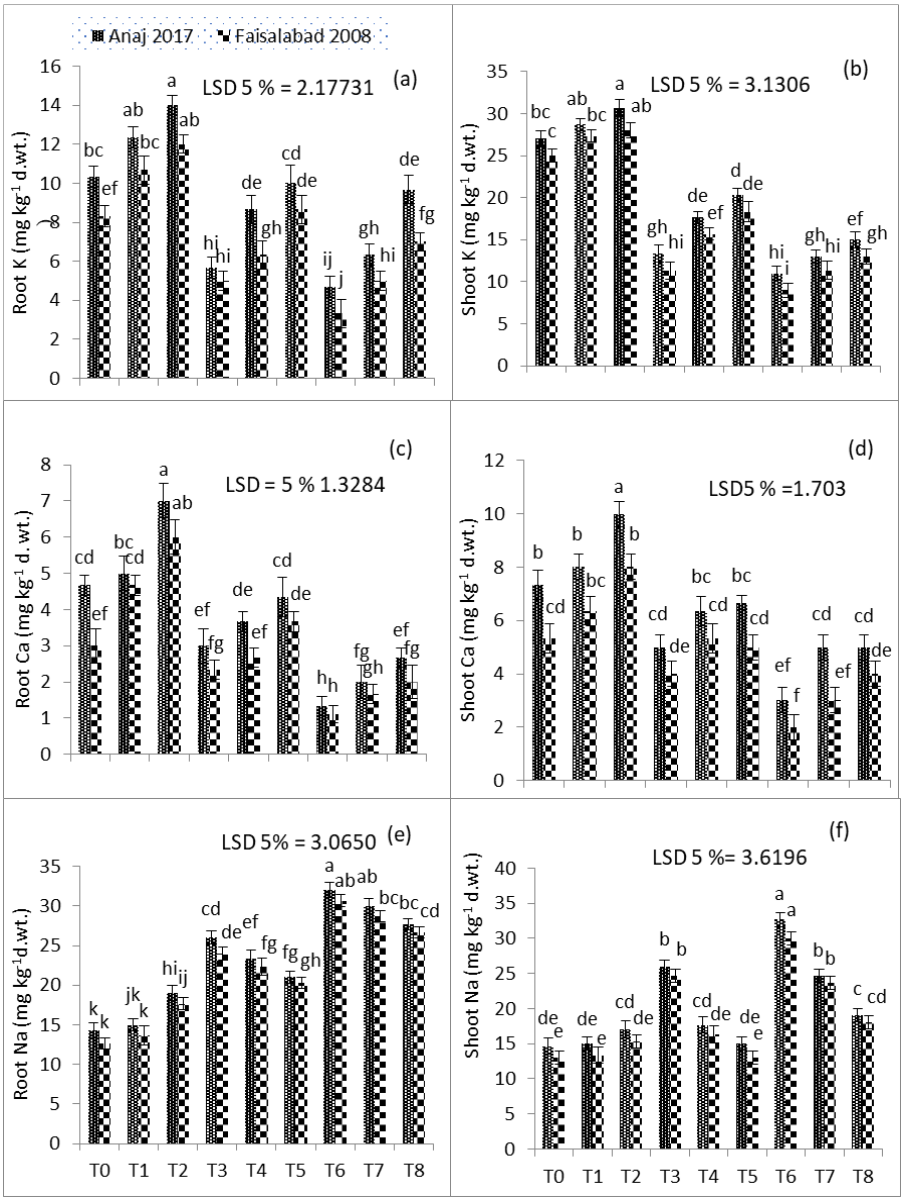

Figure 7. *Effect of Salt stress exogenously applied proline on (a) root K^+, (b) shoot K^+, (c) root Ca^{2+}, (d) shoot Ca^{2+}, (e) root Na^+ and shoot Na^+ (f) of two wheat (Triticum aestivum L.) varieties under salt stress. T_0 = Control, T_1 = 50 mM Proline, T_2 = 100 mM Proline, T_3 = 50 mM NaCl, T_4 = 50 mM NaCl + 50 mM Proline, T_5 = 50 mM NaCl + 100 mM Proline, T_6 = 100 mM NaCl, T_7 = 100 mM NaCl + 50 mM Proline, T_8 = 100 mM NaCl + 100 mM Proline*

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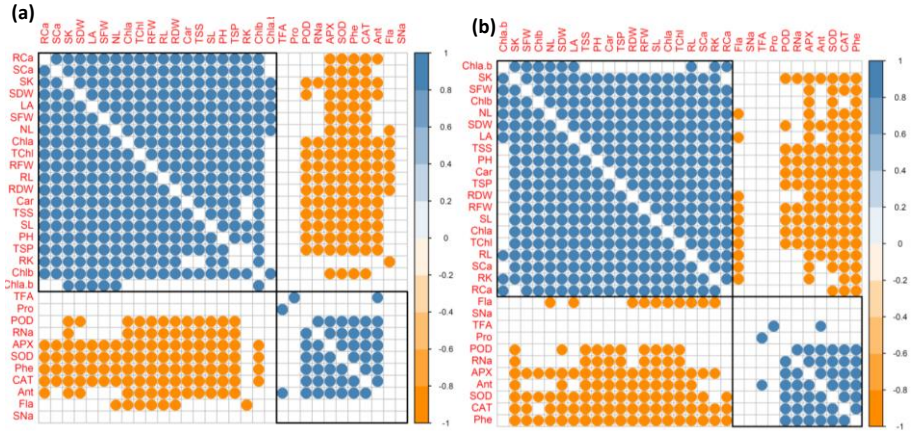

Figure 8. *A correlogram constructed for Pearson's correlation coefficient (r) showing relationship between morpho-physiological attributes of wheat varieties Aanj 2017 (a) and Faisalabad 2008 (b) in response to salt stress under exogenously applied proline. The attributes are ordered by "complete hierarchical clustering." The significance of correlation was tested (P ≤ 0.001) at 95% confidence interval. The insignificant pairs are left blank. Growth attributes: RFW: Root fresh weight; SFW: Shoot fresh weight; RL: Root length; SL: Shoot length; RDW: Root dry weight; SDW: Shoot dry weight; PH: Plant height; NL: Number of leaves per plant; LA: leaf area. Physiological attributes: Chla: Chlorophyll a; Chlb: Chlorophyll b; Car: Carotenoids; Chla.b: Chlorophyll a/b ratio; TChl: Total chlorophyll; Ant: anthocyanins; Phe: Total Phenolics; Fla: Flavonoids; Anti-oxidants: POD: Peroxidase; SOD: Super-oxide dismutase; CAT: Catalase; APX: Ascorbate per-oxidase; Osmolytes: TSS: Total soluble sugars; TSP: Total soluble proteins; TFA: Total free amino acids; Pro: Proline; Ions: RK: Root potassium; SK: Shoot potassium; RCa: Root calcium; SCa: Shoot calcium; RNa: Root sodium; SNa: Shoot sodium*

Clustered heatmap

The heatmap showed three distinct clusters for the observed wheat attributes in both genotypes. The strength of interaction was assessed on the intensities of colored rectangular blocks as shown in *Figure 9*. The hierarchies in the rows allowed for the delineation of the joint effect of distinct features was altered by various treatment levels. Almost a similar clustering pattern was observed for both cultivars Faisalabad 2008 and Anaj 2017. Growth attributes along with most of the biochemical ingredients like flavonoids, anthocyanin and phenolics, root and shoot sodium contents, activities of superoxide dismutase, ascorbate peroxide, and catalase, were grouped together in the first cluster. Here, the heatmap demonstrated the strong negative effects of the 100 mM NaCl on these attributes when no proline was applied which was correlated to lesser less degree when 50 mM proline was applied at the same salinity level. These attributes were lesser correlated to low salinity levels (50 mM NaCl) particularly at 50 mm proline level while negatively correlated at untreated control or control plants sprayed with 50 or 100 mM proline in non-saline conditions. The TFA and proline were clustered in separate cluster and all antioxidants (SOD, POD, CAT and APX), phenols, anthocyanins and flavonoids in third cluster along with root and shoot Na content. All these attributes were strong positively correlated at the highest salinity level either sprayed with proline or without it. These attributes were strong negatively correlated to all controls, i.e. those plants sprayed with 50 or 100 mM proline under non-saline conditions (*Fig. 9a, b*).

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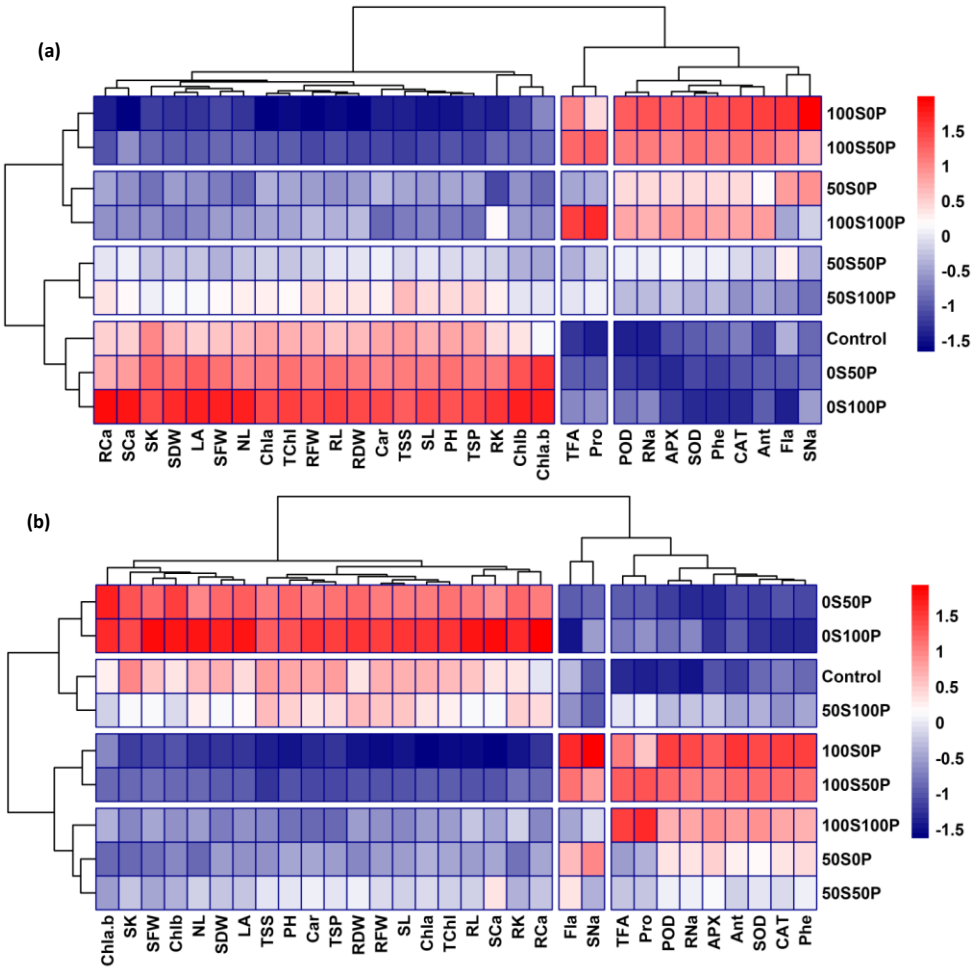

Figure 9. *Clustered heatmap for morpho-physiological attributes of wheat varieties Aanj 2017 (a) and Faisalabad 2008 (b) in response to salt stress under exogenously applied proline. Both treatments (right panel) and parameters (bottom panel) are clustered by Euclidean distance coefficients and ordered by "complete hierarchical clustering." The rows and columns are cut into slices from 2nd branch for better visualization. Treatments: Control: 0 mM NaCl + 0 mM Proline; 0S50P: 0 mM NaCl + 50 mM Proline; 0S100P: 0 mM NaCl + 100 mM Proline; 50S0P: 50 mM NaCl + 0 mM Proline; 50S50P: 50 mM NaCl + 50 mM Proline; 50S100P: 50 mM NaCl treated with 100 mM Proline; 100S0P: 100 mM NaCl + 0 mM Proline; 100S50P: 100 mM NaCl + 50 mM Proline; 100S100P: 100 mM NaCl + 100 mM Proline. Growth attributes: RFW: Root fresh weight; SFW: Shoot fresh weight; RL: Root length; SL: Shoot length; RDW: Root dry weight; SDW: Shoot dry weight; PH: Plant height; NL: Number of leaves per plant; LA: leaf area. Physiological attributes: Chla: Chlorophyll a; Chlb: Chlorophyll b; Car: Carotenoids; Chla.b: Chlorophyll a/b ratio; TChl: Total chlorophyll; Ant: anthocyanins; Phe: Total Phenolics; Fla: Flavonoids. Anti-oxidants: POD: Peroxidase; SOD: Super-oxide dismutase; CAT: Catalase; APX: Ascorbate per-oxidase. Osmolytes: TSS: Total soluble sugars; TSP: Total soluble proteins; TFA: Total free amino acids; Pro: Proline. Ions: RK: Root potassium; SK: Shoot potassium; RCa: Root calcium; SCa: Shoot calcium; RNa: Root sodium; SNa: Shoot sodium*

Principal component analysis (PCA)

The PCA analysis revealed grouping for germination, growth, and physio-biochemical characteristics of both genotypes in two distinct clusters (*Fig. 10*). The

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95.1% (89.2% along first axis and 5.4% along second axis) of the variability was explained by the first two PCA axes. Most of the recorded plants physiological attributes and growth parameters were attributed in the bigger cluster towards genotype Aanj 2017 either in control conditions and in those plants exposed to salinity stress with or without proline application. Additionally, the color scale used to express strength of variables indicated that all recorded plant attributes were strong contributors in PCA plot. The only attribute plotted towards Faisalabad 2008 was flavonoids contents plotted in the middle of 50 and 100 mM NaCl application but without proline application. Most of the biochemical parameters especially Anti-oxidants (POD, SOD, CAT and APX), osmolytes (Proline and TFA) and root and shoot Na were associated with each other at the higher salt levels (100 mM NaCl) supplied with the external proline (100 mM NaCl). It affirms that the exogenously applied proline results in the exaggerated growth of the wheat seedlings even grown under the saline medium. The genotype (Faisalabad 2008) did not produce any conclusive grouping as no growth parameters seen to be linked with each other. From the point of view of these observations, it can be concluded that Aanj 2017 responds positively to the application of proline and resists the salt stress while Faisalabad 2008 seems not to be sensitive to the externally applied salt and proline. The seedlings in the control groups were plotted apart from other treatments showing no relationship with the observed parameters.

Discussion

Salinity is one of the greatest abiotic stresses that cause a huge loss to the crops on a wide-scale globally. Excessive salt concentration in the plant damages the chemical composition of biological molecules and disturbs the metabolic processes. Pursuing the counteractive measures to salinity the effects of foliar spray of proline was evaluate in this study by examining the morpho-physiological and biochemical parameters of two wheat cultivars. Although both the genotypes showed differential growth patterns at different salinity and proline levels, however, useful aspects of proline were affirmed in control as well as in other groups.

The toxicity of the salt stress imposed enormous negative impacts on the growth performance like fresh and dry biomass, root and shoot length, number of leaves, leaf area, and concentrations of chlorophyll pigments. Salinity causes reduction in physiological attributes of plants due to accumulation of toxic chemicals following dehydration (Datta et al., 2009) and osmotic imbalance (Saddiq et al., 2021). The statistical analysis of the collected data in this study supports the mitigating role of proline against salinity and brings about significant improvements in morpho-physiological features of the seedlings. These findings coincide with other investigations on wheat where proline has been reported as osmoprotectant and up-regulated the metabolic processes (Moukhtari et al., 2020).

Plants cultivated under salt-affected media seemed to be metabolically stunted. The performance of antioxidants reduced to vulnerable limits that exaggerated the oxidative damage due to accumulation of ROS. The biochemical assays of peroxidases, catalases, and superoxide dismutase have shown to be deviated and caused stunted seedling growth under saline conditions while their activities were measured optimally in control groups and particularly under exogenously applied proline. Foliar spray of proline on the plants grown in saline conditions has

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DOI: http://dx.doi.org/10.15666/aeer/2206_59295947

tremendously improved the antioxidant activities of phenolics and other essential enzymes which assisted in counteracting the salt-stress (Hayat et al., 2013). Several other studies also confirmed the significant roles of proline in mitigating salinity by modulating the biological activities of metabolites such as upregulation of antioxidants (Huang et al., 2009).

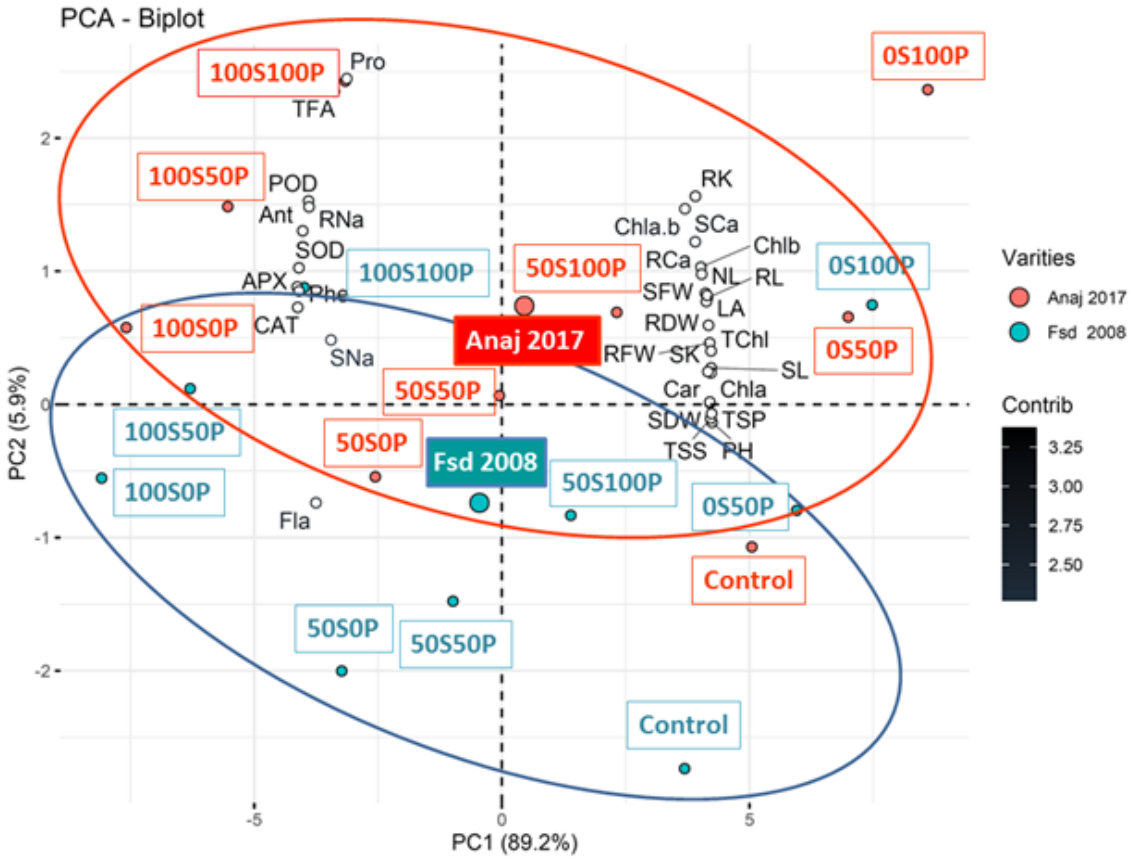

Figure 10. *A biplot constructed from principal component analysis (PCA) showing grouping of various morpho-physiological attributes of two wheat varieties Aanj 2017 and Faisalabad 2008 in response to salt stress under exogenously applied proline. The strength of plant attributes is categorized by a contribution (Contrib) scale of 2.25 to 3.75 and is reflected by color darkness in PCA Biplot. Based on eclipses drawn, Aanaj 2017 (red color) is concluded as tolerant and more responsive to proline application, and, Faisalabad 2008 (teal color) as sensitive and less responsive to proline application. Treatments: Control: 0 mM NaCl + 0 mM Proline; 0S50P: 0 mM NaCl + 50 mM Proline; 0S100P: 0 mM NaCl + 100 mM Proline; 50S0P: 50 mM NaCl + 0 mM Proline; 50S50P: 50 mM NaCl + 50 mM Proline; 50S100P: 50 mM NaCl treated with 100 mM Proline; 100S0P: 100 mM NaCl + 0 mM Proline; 100S50P: 100 mM NaCl + 50 mM Proline; 100S100P: 100 mM NaCl + 100 mM Proline. Growth attributes: RFW: Root fresh weight; SFW: Shoot fresh weight; RL: Root length; SL: Shoot length; RDW: Root dry weight; SDW: Shoot dry weight; PH: Plant height; NL: Number of leaves per plant; LA: leaf area. Physiological attributes: Chla: Chlorophyll a; Chlb: Chlorophyll b; Car: Carotenoids; Chla.b: Chlorophyll a/b ratio; TChl: Total chlorophyll; Ant: anthocyanins; Phe: Total Phenolics; Fla: Flavonoids. Anti-oxidants: POD: Peroxidase; SOD: Super-oxide dismutase; CAT: Catalase; APX: Ascorbate per-oxidase. Osmolytes: TSS: Total soluble sugars; TSP: Total soluble proteins; TFA: Total free amino acids; Pro: Proline. Ions: RK: Root potassium; SK: Shoot potassium; RCa: Root calcium; SCa: Shoot calcium; RNa: Root sodium; SNa: Shoot sodium*

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The soluble sugars are the major type of osmoprotectants which maintain the homeostatic balance of solutes and water under acclimation of saline or drought stress (Hassanein et al., 2009). Under Salinity stress, the osmoregulatory processes are diminished however exogenously applied proline triggers the carbohydrate metabolism producing metabolically active sugars which are involved in suppressing the inhibitory interactions of salinity ultimately improve the plants growth (Samad et al., 2011). In our investigation, reduction in the TSS and TSP contents in the presence of higher salts levels (100 mM) has been observed. However, their concentrations were compensated in the presence of exogenously applied higher proline levels (100 mM) in concurrence with research of Mehboob et al., 2016.

The endogenous proline and amino acids concentrations increased with the application of NaCl and exogenously applied proline. The mentioned parameters tend to be increased under high salt levels in plants which is an intrinsic osmoregulatory mechanism in plants (Miranda et al., 2014). The simultaneous influence of salinity and exogenously applied proline followed same pattern in rice (Hakim et al., 2014) and *Phragmites australis* (Xie et al., 2020). The activity of enzymes involved in amino acid biosynthesis and proline tend to increase under the availability of salt (Munns et al., 2006).

The levels of mineral ions like sodium, potassium, calcium, zinc, phosphorus have been altered to various extents under saline conditions. Sodium ion concentration tends to increase in the salt stressed treatments while K^+/Na^+ ratio reduced significantly which caused a reduction in growth parameters by reducing the stomatal conductance and water uptake. It has been revealed in several studies that salinity induces the turgor stress ion imbalance, and accumulation of toxic chemical compounds (Saddiq et al., 2021). The salinity causes irreversible loss to crops by decreasing the availability of essential metallic ions and diverting the biochemical pathways (Hamid et al., 2010). However, application of foliar spray of proline has tremendously prevented the negative effects of salinity stress by decreasing the sodium ions concentrations and increasing the K^+/Na^+ ratio which up-regulated the water relations and improved stomatal conductance and reduced the oxidative stress of ROS (Dong et al., 2017). Similar effects of salinity and proline correlations had previously been observed in corn, broad bean, and faba beans (Nessim et al., 2008; Ali et al., 2013; Dawood et al., 2014), respectively.

Conclusion

Saline soil has growth retarding effects on the crops. This study has concluded the role of proline as foliar spray on two wheat genotypes at early-stage development of seedlings. The effectiveness of the externally applied proline has been demonstrated by the improvements in morpho-physiological and biochemical parameters. Higher concentrations of proline (100 mM) proved to give best outcomes and reclaimed the inhibitory effects of salinity exclusively. Genotype Aanj 2017 tends to be salt resistant under exogenous application of proline while Faisalabad 2008 remained susceptible to the salinity stress.

Acknowledgments. The authors extend their appreciation to the Researchers Supporting Project number (RSP2024R298), King Saud University, Riyadh, Saudi Arabia.

Conflict of interest. The authors declare no conflict of interest.

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DOI: http://dx.doi.org/10.15666/aeer/2206_59295947

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